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Retinoic acid signaling is essential for formation of the heart tube in *Xenopus*

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Abstract

Retinoic acid is clearly important for the development of the heart. In this paper, we provide evidence that retinoic acid is essential for multiple aspects of cardiogenesis in *Xenopus* by examining embryos that have been exposed to retinoic acid receptor antagonists. Early in cardiogenesis, retinoic acid alters the expression of key genes in the lateral plate mesoderm including *Nkx2.5* and *HAND1*, indicating that early patterning of the lateral plate mesoderm is, in part, controlled by retinoic acid. We found that, in *Xenopus*, the transition of the heart from a sheet of cells to a tube required retinoic acid signaling. The requirement for retinoic acid signaling was determined to take place during a narrow window of time between embryonic stages 14 and 18, well before heart tube closure. At the highest doses used, the lateral fields of myocardium fail to fuse, intermediate doses lead to a fusion of the two sides but failure to form a tube, and embryos exposed to lower concentrations of antagonist form a heart tube that failed to complete all the landmark changes that characterize looping. The myocardial phenotypes observed when exposed to the retinoic acid antagonist resemble the myocardium from earlier stages of cardiogenesis, although precocious expression of cardiac differentiation markers was not seen. The morphology of individual cells within the myocardium appeared immature, closely resembling the shape and size of cells at earlier stages of development. However, the failures in morphogenesis are not merely a slowing of development because, even when allowed to develop through stage 40, the heart tubes did not close when embryos were exposed to high levels of antagonist. Indeed, some aspects of left–right asymmetry also remained even in hearts that never formed a tube. These results demonstrate that components of the retinoic acid signaling pathway are necessary for the progression of cardiac morphogenesis in *Xenopus*.

Keywords: Cardiogenesis; *Xenopus*; Retinoic acid; Nkx2.5; GATA-4; Cardiac troponin I; Heart development; Retinoic acid antagonist; Left–right asymmetry

Introduction

Primarily because of its ease of manipulation and ability to survive with a severely compromised heart, *Xenopus* has emerged as an excellent model for looking at inductive interactions in early cardiogenesis (Mohun et al., 2003). In *Xenopus*, the heart is specified during gastrulation as a result of signals emanating from Spemann's organizer and from the deep endoderm (Nascone and Mercola, 1995; Sater and Jacobson, 1989; Schneider and Mercola, 1999). After specification, the heart is located on either side of Spemann's organizer as two bilateral patches. These patches subsequently migrate and fuse at the ventral midline, just caudal to the cement gland. Once fused, the lateral edges of the sheet of cells pinch together to form a linear heart tube (Raffin et al., 2000). The heart tube then undergoes its characteristic dextral looping and further complex morphogenetic movements that result in the final three-chambered heart of *Xenopus* (Kolker et al., 2000; Mohun et al., 2000). Interplay between multiple signaling systems is required for specification and patterning of the heart, including the *wnt*

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signaling pathway (Foley and Mercola, 2005; Pandur et al., 2002; Schneider and Mercola, 2001), fibroblast growth factor signaling (Alsan and Schultheiss, 2002), bone morphogenetic protein signaling (Breckenridge et al., 2001), and the Notch signaling pathway (Rones et al., 2000).

Retinoic acid (RA) is also critical in the patterning and development of the heart. RA, the active form of vitamin A, binds to RA receptors (RARα, β, and γ) and their heterodimeric partner, members of the retinoid X receptor family (RXRα, β, and γ). This complex acts as a ligand-activated transcription factor, binding to retinoic acid response elements (RAREs) of target genes, and it is essential for many aspects of embryonic development (Glass and Rosenfeld, 2000; Ross et al., 2000; Zile, 2001). In the embryonic heart, RA signaling is primarily mediated through the RARα receptor with a minor component of the signal coming through the RARβ receptor (Kastner et al., 1997). The enzyme primarily responsible for RA synthesis, retinaldeyde dehydrogenase 2 (RALDH2), is expressed adjacent to the developing sino-atrial region during early cardiogenesis (Chen et al., 2001; Hochgreb et al., 2003; Xavier-Neto et al., 1999), suggesting that a gradient of RA may be generated along the anterior–posterior axis of the heart tube.

Embryos that have had RA signaling attenuated have a variety of cardiac phenotypes indicating multiple roles for RA in cardiogenesis. In zebrafish, embryos that lack RA signaling have an excess of cardiomyocytes indicating that RA limits the pool of cardiac progenitors in the cardiac forming region (Keegan et al., 2005). Loss of function experiments in mammalian embryos created by genetic ablation of RA receptors or RALDH2 (Niederreither et al., 2001) have shown that RA is necessary for normal cardiac morphogenesis and anterior–posterior patterning of the heart tube (Hochgreb et al., 2003; Iulianella and Lohnes, 2002; Kastner et al., 1997; Sucov et al., 1994). Early developmental defects resulting from reduced RA signaling include abnormal cardiac looping, altered sino-atrial development, and prematurely differentiated ventricular cardiomyocytes (Hochgreb et al., 2003; Niederreither et al., 2001). Exclusion of RA from the ventricle region is essential for formation of this region in early cardiogenesis (Rosenthal and Xavier-Neto, 2000; Xavier-Neto et al., 2001). In chick, loss of function experiments using dietary deficiency and by the use of RA antagonists have demonstrated anterior–posterior defects and disrupted heart looping (Chazaud et al., 1999; Ghatpande et al., 2000; Hochgreb et al., 2003; Kostetskii et al., 1999; Tsukui et al., 1999). During later stages of cardiogenesis, RA is generated by the epicardium and is important in stimulating proliferation of the ventricular myocardium (Chen et al., 2002; Munoz-Chapuli et al., 2002; Stuckmann et al., 2003; Xavier-Neto et al., 2000).

Several key questions remain concerning the role of RA in cardiogenesis. First, do the variety of roles ascribed to RA represent a common underlying theme such as axis formation or modulation of differentiation? Secondly, if the sites of synthesis and degradation of RA are arranged in such a manner that a graded level of RA should be seen in the heart, why does a global application of RA rescue cardiac defects in various models of RA insufficiency (Xavier-Neto et al., 2001)? A better understanding of the phylogenetic differences in cardiogenesis, and the role of RA signaling in the process, will help elucidate underlying mechanisms in heart development (Xavier-Neto et al., 2001).

To this end, we have endeavored to clarify the role of RA signaling in *Xenopus* cardiogenesis. As in other model systems, *RALDH2* is expressed near the sino-atrial region of the developing heart, and Cyp26, the enzyme primarily responsible for RA degradation, is expressed in a non-overlapping, complementary pattern (Haselbeck et al., 1999; Hollemann et al., 1998). *Xenopus* embryos exposed to excess RA prior to cardiac differentiation have a presumptive myocardium with reduced levels of *Nkx2.5* and increased levels of *GATA-4*, *-5*, and *-6* (Jiang et al., 1999). This treatment can result in a complete block to myocardial differentiation as assayed by cardiac troponin I (cTnI) expression (Drysdale et al., 1997). Although these gain of function experiments indicate that levels of RA signaling may also be important in amphibian cardiogenesis, these gain of function experiments do not define the normal role for RA signaling in *Xenopus* cardiogenesis.

Through the use RA antagonists (Teng et al., 1997), we demonstrate for the first time that the RA signaling pathway is essential, during a restricted window of time after gastrulation, for early events in cardiac morphogenesis in *Xenopus*. The primary defect is an inability to form a heart tube from the original sheet of cells. At lower concentrations of the antagonist, cardiac morphogenesis proceeds further but is still blocked before normal morphogenesis is completed. If embryos are treated with RA antagonist prior to gastrulation, the *Nkx2.5* expression domain is expanded, as observed in zebrafish (Keegan et al., 2005), but this initial increase in the size of the cardiac domain is not sustained. Despite the altered appearance of the myocardium, we do not find that reduced RA signaling leads to premature differentiation of the myocardium contrasting observations in mice, although the myocardium remains immature in appearance. We also demonstrate that the myocardium still has some of the characteristics of left–right asymmetry, although many of the characteristic shape changes that occur during looping were clearly absent. Thus, we provide evidence that stage-specific RA signaling is critical for completion of the cardiac development in *Xenopus*.

Materials and methods

Embryo collection

Female *Xenopus laevis* frogs were injected with 600–700 IU of human chorionic gonadotrophin (Sigma) to induce ovulation. In vitro fertilization of ovulated eggs was performed in 80% Steinberg's solution containing minced testis. Embryos were dejellied with 2.5% cysteine, pH 8.0, and cultured in 20% Steinberg's solution. Embryos were staged according to Nieuwkoop and Faber (1994).

Experimental treatments

Experimental treatments included exposure to 1 μM all-*trans* retinoic acid (RA, Sigma), 1 μM retinoic acid receptor α agonist (Allergan #193836, Teng et al., 1996), pan-retinoic acid receptor antagonist (Allergan #193109, Johnson et al., 1995), and 1 μM retinoic acid receptor α antagonist (Allergan #194301,

Teng et al., 1997) in 20% Steinberg's. Solutions were made from 1 mM stock solutions in 100% ethanol or DMSO; as a result, the control treatment was 0.1% ethanol or DMSO in 20% Steinberg's. Treatments were done by immersion and were continuous until the embryos were fixed for in situ hybridization or confocal microscopy.

In experiments which determine the timing of differentiation events, batches of embryos were treated with either 1 μM AGN 194301 or 1 μM RA and embryos from each batch were assayed for cardiac differentiation by whole-mount in situ hybridization for *cTnI* or *αMHC* mRNA.

Whole-mount in situ hybridization and histology

Whole-mount in situ hybridization was performed according to Harland (1991) with modifications by Drysdale et al. (1997). In addition, embryos were blocked using a maleic acid buffer (pH 7.5), 2% Boehringer Mannheim blocking reagent, and 4% heat-treated lamb serum solution and washed in maleic acid buffer. Antisense riboprobes for *Nkx2.5* (Tonissen et al., 1994), *GATA-4* (Kelley et al., 1993), *cTnI* (Drysdale et al., 1994), and αMHC (Logan and Mohun, 1993) were labeled with dioxygenin-UTP (Roche Diagnostics) according to Harland (1991) except that ^{32}P-labeled nucleotides were not incorporated into the probes. Probes were re-suspended directly in RNA hybridization buffer without alkaline hydrolysis. Once the color reaction was complete, using NBT/BCIP or BM Purple (Roche Diagnostics), embryos were fixed overnight in Bouin's fixative, and endogenous pigment was bleached out under fluorescent light, using a solution containing 1% hydrogen peroxide, 5% formamide, and 0.5× SSC, for several hours. To better see internal structures, some embryos were cleared in 1 benzyl alcohol:2 benzyl benzoate. Embryos were photographed using a Leica MZ12 dissecting microscope and Northern Eclipse software (Empix Imaging).

For sections, embryos were fixed according to the whole-mount procedure but were then re-hydrated after storage in methanol in a methanol series, rinsed in PBS, and incubated in 15% sucrose in PBS. Embryos were then placed in embedding solution (15% sucrose, 7.5% gelatin in PBS) overnight. Embryos were then frozen on dry ice and sectioned on a Leica CM1900 cryostat microtome. Cryostat sections of embryos were cut at 15 μm. In situ hybridization on the slide was then done using standard procedures (Jensen and Wallace, 1997).

Heart rate analysis

Heart rate was calculated based on the number of heart contractions in a 10 s period, with 40 individual embryos measured for each treatment. Measurements of beating heart rate are presented as mean ± SEM in beats/minute. Statistical significance differences were calculated using a student's *t* test. Significance was accepted at the level of $P < 0.01$.

Confocal microscopy

Tadpoles were fixed in Dent's Fixative (80% methanol, 20% dimethyl sulfoxide (DMSO)) and stored at −20°C until processed. Except where noted, subsequent treatments and rinses were carried out on samples in 24-well plates on an orbital shaker. Prior to immunolabeling, the ventral dermal layer overlying the hearts was manually removed to help antibody penetration and ensure optimal imaging of the hearts. Embryos were rehydrated to PBS in a methanol–PBS series which included 1% DMSO at each step and blocked for 4 h at room temperature or overnight at 4°C in PBS–TD blocking solution (PBS, 1% Tween 20, 1% DMSO, 0.02% NaN_3) containing 0.1 M glycine, 2% powdered milk, and 1% BSA. Primary antibodies were diluted with block solution. The cardiac troponin I antibody, purchased from Fitzgerald, was diluted 1:500; the flectin (F-22) antibody, a gift from Nancy Philp (Thomas Jefferson University), was used at 1:40. Incubation with primary antibody was carried out overnight at 4°C. Samples were then rinsed six to eight times over 8–10 h with PBS–TD. Secondary antibodies were used as follows: anti-goat conjugated to Alexa 568, anti-mouse IgG conjugated to Cy5 (both from Jackson Immunoresearch). Both were diluted 1:200 in blocking solution and allowed to incubate with the sample overnight at 4°C. Samples were rinsed as described for primary antibody treatment and subsequently dehydrated in an ethanol series. Embryos were mounted for viewing as described in Kolker et al. (2000).

Results

Retinoic acid signaling is required for formation of the heart tube

By stage 30/32, the *Xenopus* heart has differentiated, as detected by expression of *cTnI* or *αMHC* (Figs. 1A, B). As

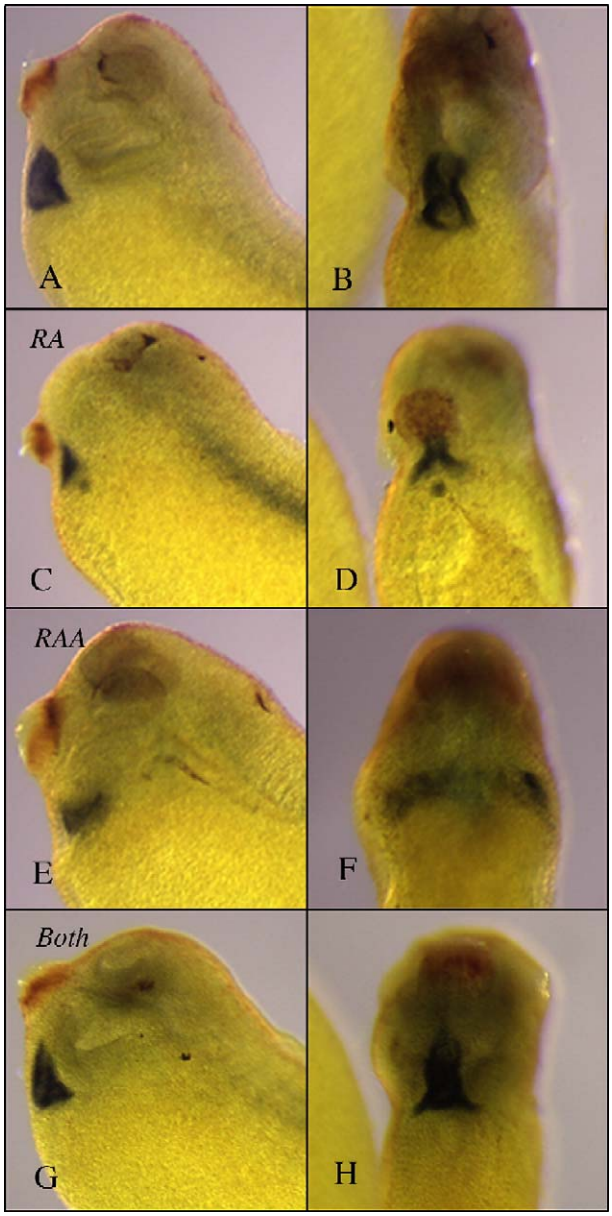

Fig. 1. Blocking retinoic acid signaling at stage 12/14 results in a failure to form a simple heart tube. Whole-mount in situ hybridization for *cTnI* was used to visualize the differentiated myocardium (blue staining). In stage 31 embryos, a normal linear heart tube that is just starting to loop (A). The tube and looping are best viewed from the ventral side of the embryo (B). When embryos are treated with 1 μM RA, the heart is severely disrupted (C), although when viewed from the ventral side, it can be seen that a tube is still formed (D). When treated with 1 μM AGN 194301, myocardial differentiation still occurs (E), but when viewed from the ventral side, the lack of tube formation is clear (F). Addition of equimolar (1 μM) concentrations of both RA and AGN 194301 resulted in an apparently normal heart tube indicating that the observed effects are not due to toxicity of the compounds used (G, H).

expected from our previous studies (Drysdale et al., 1994, 1997; Jiang et al., 1999), continuous treatment with 1 μM all-*trans* RA from stage 12 until stage 30/32 resulted in either a marked inhibition or complete elimination of *cTnI* expression (Figs. 1C, D). In these assays, *cTnI* expression is used as an indicator of myocardial differentiation. The region of *cTnI* expression was markedly reduced, when present, and located in a more anterior position than in control embryos. Despite the substantial reduction in size, heart tube closure is observed (Figs. 1C, D).

We contrast the changes above with those resulting from treatment with 1 μM RA antagonist (AGN 194301). Embryos treated with the antagonist starting at stage 12/14 displayed a moderate inhibition of the *cTnI* expression domain. However, the expression domain that remained was very different from the *cTnI* expression domain in either control embryos or those treated with RA. While the RA-treated embryos retained the anterior expression of *cTnI*, embryos treated with AGN 194301 retained expression at the posterior end of the normal heart-forming region (Figs. 1E, F). The hearts in these embryos never formed a tube. Instead, the myocardium appeared as a sheet of cells on the ventral side of the embryo. Furthermore, in embryos treated with 1 μM AGN 194301, the *cTnI* expression domain is often split with an apparent failure to meet properly along the ventral midline, retaining a more lateral position. These changes in cardiac morphology were not due to toxic effects as adding both 1 μM RA and 1 μM AGN 194301 to embryo cultures largely restored normal cardiac development (Figs. 1G, H).

As AGN 194301 is an RARα antagonist, it is possible that blocking this specific receptor may cause increased signaling to the other RAR family members, although at a concentration of 1 μM, AGN 194301 will likely inhibit other RARs. To eliminate this possibility, we also treated embryos with AGN 194109, a pan RAR antagonist (Johnson et al., 1995). We could not detect any differences in phenotype between embryos treated with AGN 194301 and AGN 194109-treated embryos when used at similar concentrations and times. Thus, we conclude that the observed phenotype when adding AGN 194301 is due to an attenuation of RA signaling.

Addition of the RA antagonist, AGN 194301, at stage 12/14 resulted in few obvious morphological changes to the rest of the embryo. A modest increase in the size of the head and the cement gland was observed (Fig. 1E), and the embryos appeared to lack lymph hearts, a small beating organ found near the developing kidney (data not shown), which had previously been shown to increase in size as a result of RA treatment (Drysdale et al., 1994).

Embryos continuously treated with AGN 194301, from stage 14 onwards, were sectioned and stained over the length of the developing heart. The failure to form a tube is seen along the entire length of the heart tube. The degree of heart tube formation was concentration-dependent (Fig. 2). At 1 μM AGN 194301, there was no evidence of any movements towards generating a tube (Figs. 2B, F, J, N, R). In embryos that were treated with 100 nM AGN 194301 (Figs. 2C, G, K, O, S), the two sides of the patches did appear to be, at least superficially, trying to form a tube. At the 10 nM AGN 194301 dose, there was partial closure of the heart tube (Fig. 2L), but, at stage 32/34, the heart tube still did not appear to have undergone any looping morphogenesis.

The effects of AGN 194301 on heart formation were best visualized by looking on the ventral side of embryos (Stage 32/34) after in situ hybridization for *cTnI* (Fig. 3). At high concentrations (1 μM), AGN 194301 treatment resulted in the appearance of gaps at the ventral midline with the two patches of cardiac tissue on either side of the gap (Fig. 3B). If left to develop, the embryos only exhibit a single beating mass of tissue, so the presence of the gap did not result in obvious cardia bifida. At concentrations as low as 10 nM AGN 194301, there were still embryos where the heart remained as a ventral sheet (Fig. 3C). If the sheet was able to curl into a tube, the tube still appeared linear, indicating that looping of the heart tube was either impaired or delayed (contrast Figs. 3A and D). A summary of the cardiac morphological defects resulting from differing concentrations of AGN 194301 is listed in Table 1.

Mice that are deficient in RA signaling show premature differentiation of ventricular myocytes (Kastner et al., 1997; Niederreither et al., 2001). This could potentially explain the phenotypes observed when blocking RA synthesis. If myocardial differentiation was occurring early in *Xenopus* embryos treated with AGN 194301, then one might expect incomplete morphogenesis. To test if premature differentiation was occurring in our embryos, we assessed the timing of initial *cTnI* expression as an indicator of differentiation. In untreated embryos, cTnI is first expressed between stages 26 and 28. We found that in embryos treated with AGN 194301 did not express *cTnI* any earlier than control embryos, indicating that they were not differentiating prematurely (Table 2).

The retinoic acid signaling pathway is required between stage 14 and 18

We found that embryos continuously treated with AGN 194301, starting at stage 14, failed to form a heart tube. If the AGN 194301 treatment was initiated later than stage 16, a tube is formed, although other morphological defects were still observed. If both RA and AGN 194301 were added at the same time, heart tube formation was rescued (Figs. 4D, J), as it was if the addition of RA was delayed until stage 16 (Figs. 4E, K). However, if addition of RA was delayed until stage 18/20, a time of approximately 4 h at room temperature, formation of the tube was impaired (Figs. 4F, L). In all cases, embryos were allowed to develop until stage 31/32 where the degree of heart tube formation was assessed.

The expression patterns of Nkx2.5 and GATA-4 are altered by a lack of retinoid signaling

Nkx2.5 and *GATA-4* are both key transcription factors in cardiac development and are known to be altered by addition of RA (Jiang et al., 1999). It was therefore hypothesized that AGN 194301 treatment would also alter the expression

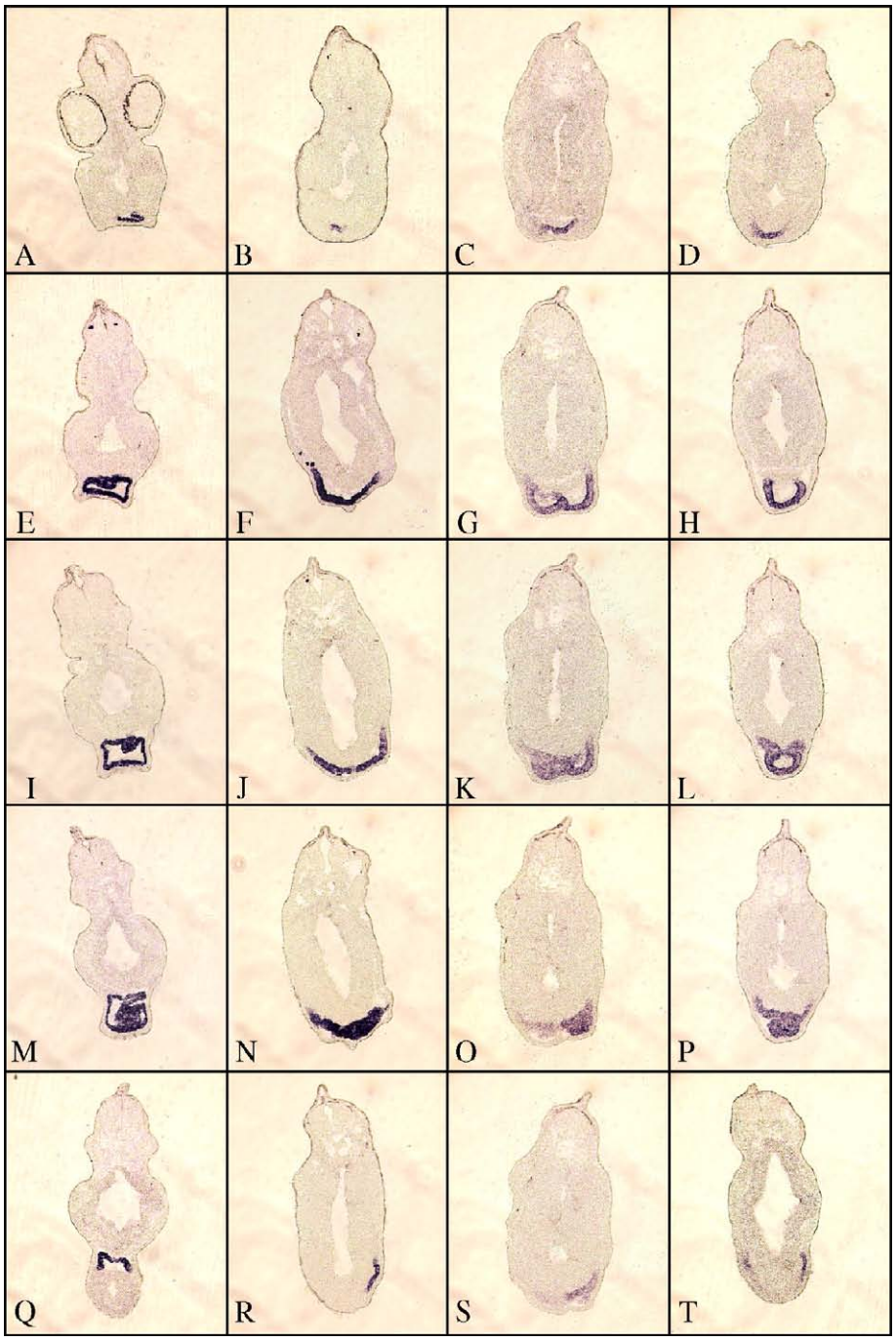

Fig. 2. Dose-dependent defects in cardiac morphogenesis caused by the retinoic acid antagonist. To better visualize the defects in cardiac morphogenesis due to addition of antagonist, *αMHC* expression was examined in serial sections along the anterior–posterior axis of stage 32/34 embryos subjected to varying amounts of AGN 194301. A complete tube with distinct left–right asymmetry was clearly evident throughout most of the control myocardial domain (first column, A, E, I, M, Q with anterior at the top and posterior at the bottom). Consistent with the whole-mount data, no evidence of linear heart tube formation was detected in 1 μM AGN 194301 treatments (second column, B, F, J, N, R), while a partial, relatively symmetrical heart tube could be detected within 100-nM-treated embryos (third column, C, G, K, O, S). Formation of a heart tube was observed in embryos subjected to 10 nM AGN 194301 (fourth column, D, H, L, P, T), although the fusion was not as extensive as seen in control embryos. In addition, at this stage, the left and right side appeared symmetric when compared to control embryos.

pattern of these key genes in a manner opposite to treatment with RA. In normal stage 30/32 embryos, *Nkx2.5* is strongly expressed in the newly formed linear heart tube and in the overlying mesocardium and pericardium (Raffin et al., 2000). Treatment with 1 μM all-*trans* RA at the end of gastrulation (stage 12) resulted in severe inhibition of *Nkx2.5* expression (Figs. 5E, F), and the staining was restricted to the anterior end of the heart tube. In a minority of cases, *Nkx2.5* expression was completely abolished.

RA antagonism with 1 μM AGN 194301 at stage 14 did not result in an expansion of the *Nkx2.5* expression domain. Instead, a moderate restriction of the *Nkx2.5* expression domain was noted, although the expression was expanded dorsally in these embryos. The spur-shaped dorsal expansion was variable

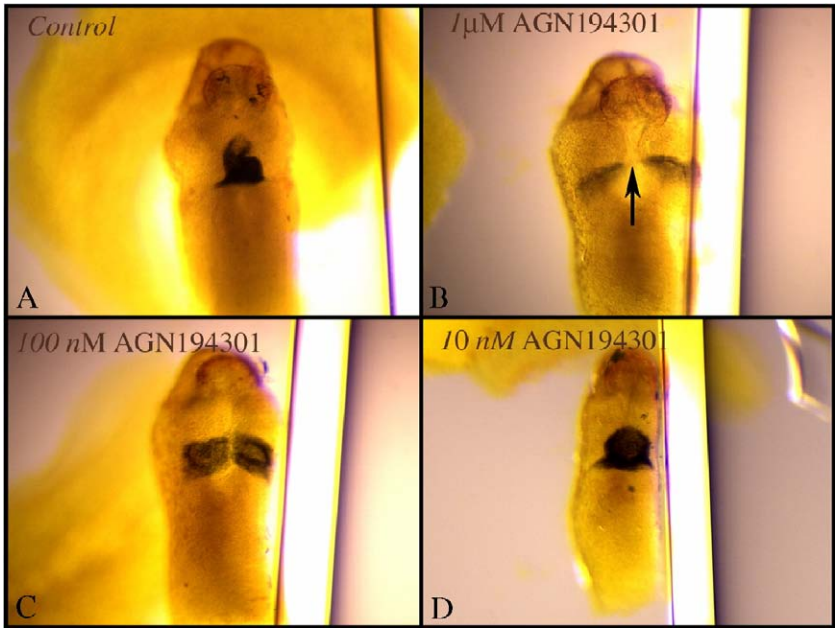

Fig. 3. Common morphologies of differentiated hearts from embryos treated with retinoic acid antagonists. When viewed from the ventral side, control embryos stained for *αMHC* expression by whole-mount in situ hybridization show a heart tube that is initiating the normal looping process (A). The most severe phenotype observed with AGN 194301 treatment was a lack of both tube formation and a ventral gap in the *αMHC* expression domain indicating a lack of fusion of the two heart primordia (B). Embryos that showed a fusion of the heart primordia at the ventral midline but still no tube formation (C) were seen with lower doses of antagonist. At even lower doses of antagonist, the most common heart phenotype observed was an apparent heart tube that appeared to not undergo looping at this stage (D).

in size but could extend to the level of the somites, ending in close proximity to the presumptive kidney (Figs. 5I, J). When antagonist-treated embryos were viewed from the ventral side, *Nkx2.5* expression was in a ventral sheet as shown for *cTnI*. Addition of equal amounts of both AGN 194301 and RA resulted in a rescue of phenotype observed for either individual treatment (Figs. 5M, N).

During heart formation, *GATA-4* is broadly expressed in the heart and upper gut region, overlapping with much of the *Nkx2.5* expression (Figs. 5C, D). Treatment with 1 μM all-*trans* RA appeared to slightly expand the expression domain of *GATA-4* and shifted the expression domain to a more anterior position beneath the cement gland (Figs. 5C, D), although it must be noted that the small changes in the head morphology that are also observed may have caused the shift in expression pattern.

The treatment with 1 μM AGN 194301 at stage 12/14 resulted in an inhibition but not elimination of *GATA-4* expression. The cells that do express GATA-4 are restricted to posterior and ventral position in the presumptive heart tube (Figs. 5K, L). The cells expressing *GATA-4* form an unfolded sheet at the time control embryos or RA-treated embryos have a heart tube. The phenotype seen when treated with the antagonist was rescued by addition of an equimolar concentration of RA (Figs. 5O, P).

Recently, similar experiments in zebrafish have shown that RA signaling can restrict the number of cardiac progenitors (Keegan et al., 2005), thus it remained possible that RA was also influencing heart tube formation and *Nkx2.5* expression earlier than stage 14. To investigate this possibility, embryos were exposed to 1 μM AGN 194301 before gastrulation (stage 6). As observed in zebrafish, early treatment with RA antagonists appeared to increase the expression domain of *Nkx2.5* (Fig. 6), when assayed before heart tube formation, although the increase was modest compared to the changes seen in zebrafish. However, if allowed to develop further, the expanded *Nkx2.5* domain was not sustained, and, by stage 32,

Table 1
Types of disruptions in cardiac morphology seen in varying concentrations of AGN 194301 (RAA)

Treatment (*n*)	Normal heart tube	Ventral gap	Ventral sheet	Symmetrical tube
1 μl/ml DMSO (22)	100	0	0	0
1 μM RAA (22)	0	45	55	0
500 nM RAA (20)	10	0	50	40
100 nM RAA (24)	8	0	34	58
50 nM RAA (28)	7	0	20	73
10 nM RAA (24)	33	0	17	50
1 μM RAA and RA (24)	92	0	0	8

Values are expressed as percentages of total embryos examined.

Table 2
Blocking retinoic acid signaling does not result in precocious expression of *cTnI*

Stage	Control	RAA-treated
24	0/34	0/45
25	0/70	0/48
26	7/52	3/52
27	27/48	9/49
28	32/36	9/24

The numbers represent the number of embryos with *cTnI* expression as detected by whole-mount in situ hybridization over the total number of embryos examined for *cTnI* expression at that stage.

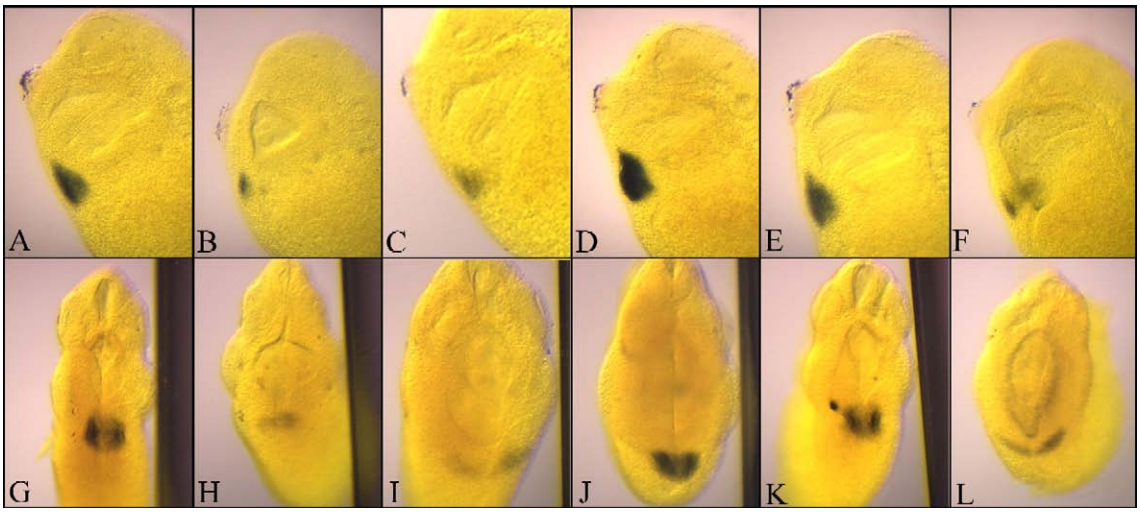

Fig. 4. Blocking RA signaling is required in a narrow window of time for formation of the heart tube. The heart has been visualized by whole-mount in situ hybridization for *cTnI* and is viewed in whole cleared embryos in side view (top panel) and from the anterior end (bottom panel) in order to visualize the formation of a tube. Normal heart tube formation can be seen in stage 31/32 embryos that were treated with 2 μl/ml DMSO as a carrier control (A, G). Treatment with 1 μM RA at stage 14 resulted in a dramatic decrease of myocardial differentiation (B, H). Treatment with 1 μM AGN 194301 resulted in a block to heart tube formation remained as a sheet along the ventral midline (C, I). *cTnI* expression levels and spatial distribution were restored if RA and AGN 194301 were given in equimolar amounts (D, J). When embryos were subjected to an initial treatment of 1 μM AGN 194301 at stage 14 with subsequent addition of 1 μM RA at stage 16, *cTnI* expression (E, K) was also similar to control patterns. However, when the addition of RA was delayed until stage 18/20, cardiomyocyte differentiation was shown to be again restricted to posterior regions of the normal heart field and linear heart tube formation was not detected (F, L). Thus, blocking RA signaling in a tight window of time between stages 14 and 18 is sufficient to prevent formation of the heart tube.

the *Nkx2.5* expression in hearts appeared similar to embryos where RA antagonism was initiated at stage 14. This was not simply a loss of *Nkx2.5* expression as the expression of cTnI was also markedly reduced (Figs. 6E, F). In zebrafish, expression of *eHAND* was used as general marker of lateral plate mesoderm, and the lack of RA signaling did not alter this expression domain, indicating that the overall size of the lateral plate mesoderm was not altered by RA antagonism. In zebrafish, there only appears to be one HAND gene (Yelon et al., 2000), and, in *Xenopus*, the expression of *HAND1* (*eHAND*) appears to most closely resemble the zebrafish pattern (Angelo et al., 2000). To further test for species-specific differences, we examined the expression *HAND1* in embryos treated with AGN 194109. The *HAND1* domain was smaller and appeared truncated at the anterior and posterior end in embryos treated with AGN 194109 (Figs. 6C, D).

Late stage consequences of altered heart morphology

Despite the vastly different morphologies of the RA and antagonist-treated embryos, the majority of embryos still formed beating tissue. When compared to normal hearts (Fig. 7A), RA-treated hearts (Fig. 7C) at stage 45 appeared smaller. There was a small but significant reduction in heart rate in these embryos. This small difference in heart rate was confirmed using AGN 193836, an RARα agonist (Fig. 7D), which again showed a small but significant drop in heart rate (Fig. 7E). Blood was seen moving through the vasculature of both RA and agonist-treated embryos, indicating that a competent cardiovascular network had been formed.

Treatment with 1 μM AGN 194301 resulted in a slow beating nub. The nub can be seen within a large cavity, which has formed below the head in the ventral half of the embryo (Fig. 7B). The nub might have been mistaken for neighboring gut tissue, except for its ability to contract. Pooling of red blood cells in the gut cavity was also observed indicating that the heart never formed a closed tube. Heart rate in these embryos was much lower than in either RA-treated or control embryos, essentially halved as a result of antagonist treatment (Fig. 7E).

Blocking RA signaling caused a failure to form a competent cardiovascular network. To determine if this was due to the lack of heart tube closure being permanent, we examined cardiac morphology at later stages of development. The resolution needed to examine this question was provided by whole-mount immunocytochemistry followed by imaging with a confocal microscope. We examined control and treated embryos that were allowed to develop until the control embryos were at stage 39. Two antibodies were used to stain the heart, anti-cardiac troponin I, and the monoclonal antibody F-22 which stains the extracellular matrix protein flectin. Flectin is an extracellular matrix protein found in the developing heart and is expressed in an asymmetrical manner (Linask et al., 2002; Tsuda et al., 1996; Kolker and Weeks, unpublished). We observe flectin in the matrix between the endocardium and myocardium as well as around the vasculature. One characteristic of myocardial development is the change in myocardial cell shape during cardiogenesis, from cells that have a distinctive columnar appearance to cells with a more compacted morphology. This progression can be seen in Figs. 8A–C. Secondly, myocardial production of flectin has a distinctive temporal progression, with staining first appearing intercellular and near the surface of the myocardium (compare Figs. 8B and C). When embryos were treated with 1 μM AGN 194301, the morphology of the

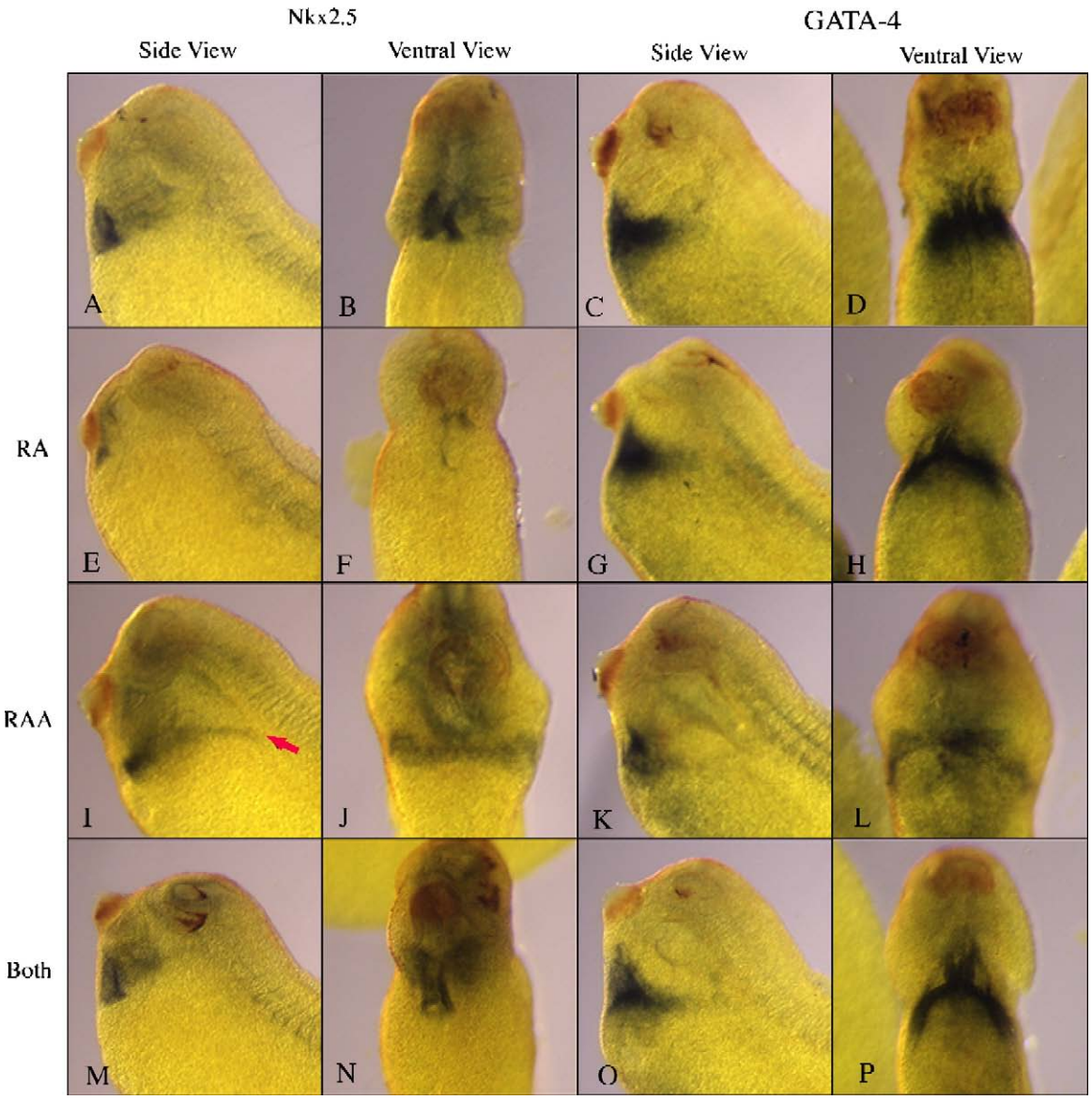

Fig. 5. Treatment with retinoic acid antagonist alters the expression domains of *Nkx2.5* and *GATA-4*. The expression domains of *Nkx2.5* and *GATA-4* are visualized by whole-mount in situ hybridization. In control embryos treated with 2 μl/ml DMSO (top panel, A–D), the normal expression domain of *Nkx2.5* and *GATA-4* can be seen in side view and viewed from the ventral side. Treatment with 1 μM RA caused a severe reduction in the expression pattern of *Nkx2.5* (E, F) but did not cause any obvious change in the expression pattern of *GATA-4* (G, H). Treatment with 1 μM AGN 194301 caused a marked change in the expression domain of *Nkx2.5*. As expected, the expression domain was restricted to a sheet of cells rather than a tube, and the expression was restricted to the posterior end of the region that normally expresses *cTnI* (I, J). In addition, there was a marked spur of *Nkx2.5* expression that extended dorsally close to the level of the somites (red arrow). Treatment with AGN 194301 caused a reduction, but not elimination of the *GATA-4* expression domain (K, L). If embryos were simultaneously exposed to 1 μM RA and 1 μM AGN 194301, the expression domains of *Nkx2.5* and *GATA-4* were similar to controls (M–P).

myocardial cells was still columnar at stage 39 (Fig. 8D), similar to cells of a control embryo at stage 31 (Fig. 8A) rather than the compact shape found in control stage 39 embryos (Fig. 8C). The non-fused lateral fields are readily visible as is the failure to form a tube, so these two features of AGN 194301 treatment have not recovered with time, however, at the later stages, the fields are not strictly a flat sheets of cells. A pouch forms from the sheet. This out-pouching occurs even if the heart forms two separate sheets of myocardial tissue that do not meet at the midline (Fig. 8D).

Even though the myocardium looks immature, it still seems able to carry out the temporally correct synthesis of flectin and transport the flectin out of the cell. Thus, the flectin staining in AGN-194301-treated embryos suggests that the myocardium was able to complete part of the cardiac molecular program even when unable to form a closed heart tube (Fig. 8).

The hearts of AGN-194301-treated embryos at stage 30/32 (Fig. 3) appeared symmetrical when observed using whole-mount in situ hybridization. This was not a surprise as disruption in left–right patterning by blocking retinoid signaling has been described in other animal models (Chazaud et al., 1999; Niederreither et al., 2001; Wasiak and Lohnes, 1999). However, left–right patterning, including looping, is a multi-

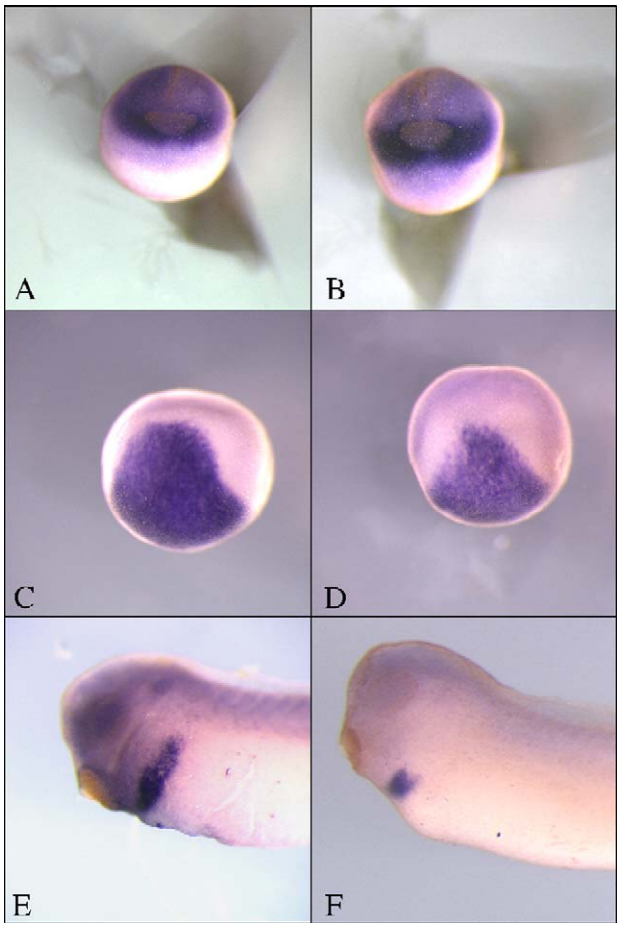

Fig. 6. Treatment of embryos with antagonist prior to gastrulation alters patterning of cardiac genes. At embryonic stage 20, *Nkx2.5* is normally expressed in a broad sheet on either side of the cement gland (A). When embryos were treated with AGN 194301 prior to gastrulation (stage 6), this domain appeared expanded, particularly along the anterior–posterior axis (B). When compared to expression in control embryos (C), the expression of the lateral plate mesoderm marker, *HAND1*, was diminished by the same treatment (D) with shortening both from the posterior and anterior end of the embryo (anterior is left and posterior is right in both C and D). Despite the early changes in *Nkx2.5* expression, the myocardium still failed to form a tube and the *Nkx2.5* (E) and *cTnI* (F) expression domain was reduced with essentially the same phenotype as observed in embryos treated with RA antagonists post-gastrulation (see Fig. 1).

step process. Although overt changes that characterize full bending of the cardiac tube were not seen, we have looked at AGN-194301-treated embryos for earlier indications that left–right patterning was still established. Confocal analysis of treated embryos suggests that at least early features of left–right patterning still occurred (Fig. 9). In normal hearts, at stage 33/34, distinct changes can be seen in the movements of heart looping. In particular, there is an inflexion on the right side that is at the anterior end of the heart tube, while an inflexion on the left side is at the posterior end. In embryos treated with AGN 194301, although no tube forms, the characteristic differences in inflexion points still occur, albeit at later stages of development. This implies that aspects of the left–right information may still be in place and also that some of the movements of looping can still occur despite not forming a tube.

Discussion

Xenopus embryos treated with RA have severe disruptions in cardiac development (Drysdale et al., 1994, 1997; Jiang et al., 1999). Those results do not imply a normal function for RA in *Xenopus* cardiogenesis. This study demonstrates that there is a specific requirement for RA signaling in *Xenopus* cardiogenesis, consistent with observations in both avian and mammalian systems, where endogenous RA signaling has been attenuated either by dietary restriction, knockouts of specific elements of the RA signaling pathway, or by use of RA antagonists (Chazaud et al., 1999; Hochgreb et al., 2003; Iulianella and Lohnes, 2002; Keegan et al., 2005; Kostetskii et al., 1999; Liberatore et al., 2000; Rosenthal and Xavier-Neto, 2000; Yutzey et al., 1995; Zile et al., 2000). Here, we describe the requirements for RA signaling in *Xenopus* cardiogenesis and demonstrate that different activities are observed depending on the precise stage of embryogenesis.

Initial roles for retinoic acid in heart development

The heart is derived from anterior lateral plate mesoderm. In zebrafish, the initial events in cardiogenesis are influenced

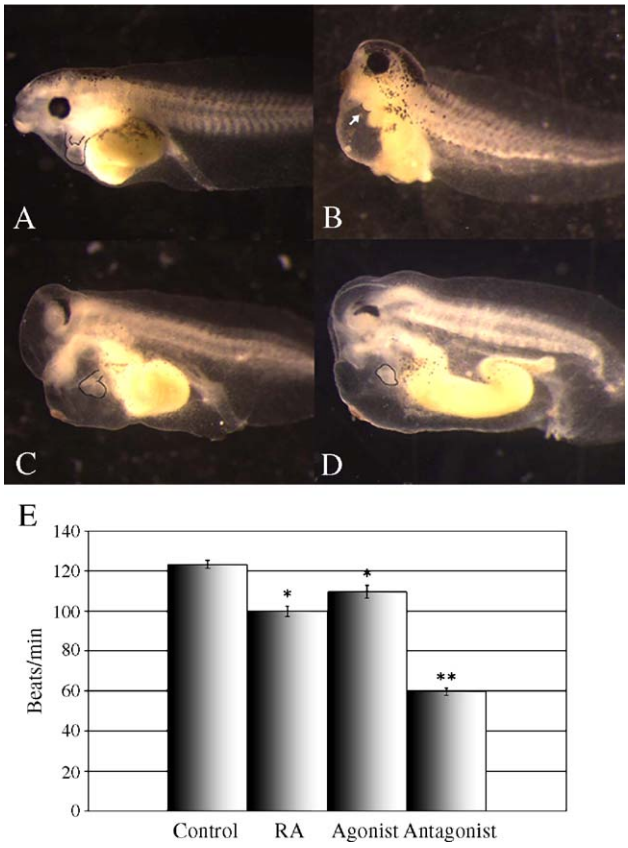

Fig. 7. Altering retinoic acid signaling has profound affects on the final morphology of the cardiovascular system. A normally developed was seen in stage 41 control embryos (A). Treatment with AGN 194301 resulted in a small contractile nub (B). Treatment with RA resulted in a smaller then normal contracting heart (C). Treatment with an agonist to RA results in a phenotypic heart similar to that produced by RA treatment (D). All hearts were outlined, in a relaxed phase, to better visualize heart size. (E) Altering RA signaling during early heart development results in a decreased heart rate in stage 41 embryos ($n = 20$, $P \leq 0.01$).

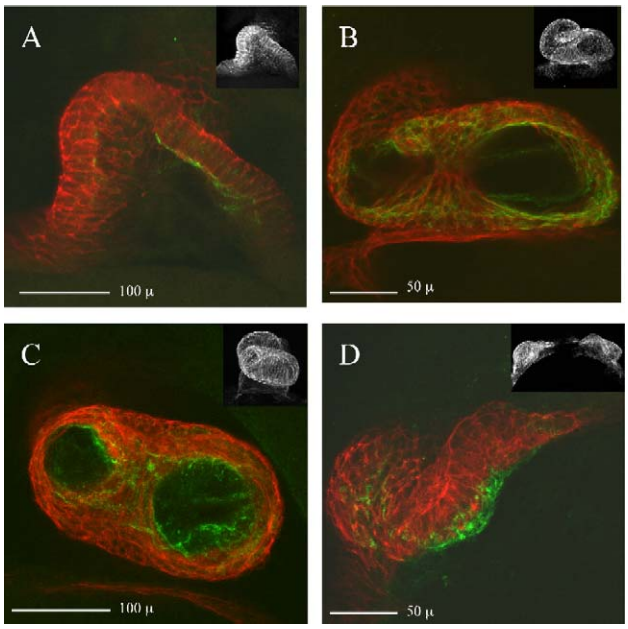

Fig. 8. Cardiomyocytes retain an immature appearance after AGN 194301 treatment. Control embryos at stage 31 (A), stage 35 (B), stage 39 (C), and an AGN-194301-treated embryo (D) fixed at the same time as control sibling shown in C were immunolabeled and analyzed by confocal microscopy (10 RA antagonist embryos were analyzed; this embryo represents the trend seen). The black and white inset represents a compilation of all the optical sections of a *z*-series (60–70 4 μm sections) taken through the embryos as viewed with the muscle marker cardiac troponin I. The colored micrograph represents an optical thick section that is a subset of the inset (10 consecutive sections). Red = cardiac troponin I; green = F-22, flectin. The normal stage 31 embryo (A) shows the heart region after the left and right fields have fused but prior to tube closure. The troponin I staining reveals large columnar muscle cells characteristic of the stage. There is some flectin staining apparent on the basal side of the mesoderm as well as faint intracellular staining in the muscle cells of the left heart field in this particular subset of sections. At stage 35 (B), the heart has fused to form a tube and is beginning to undergo S-shaped looping. The muscle cells have lost their columnar appearance, and muscle striations are visible. Flectin is distributed in a subset of muscle cells as well as both the basal and apical surface of the endocardium. The normal stage 39 embryo heart (C) is viewed just prior to trabeculation; the striations more evident. The majority of the flectin staining is seen in the ECM. The 1 μM AGN-194301-treated embryos have unfused heart fields. They have not formed tubes but as they develop do not remain as flat sheets. The muscle cells have the columnar appearance of a less mature stage 31 embryo. The flectin staining has a temporal characteristic more similar to stage 39 with the majority of the localization in the ECM.

by RA as it restricts the number of cardiac progenitor cells within the lateral plate mesoderm (Keegan et al., 2005). A similar process occurs in *Xenopus* because, when RA signaling is blocked prior to gastrulation, the early *Nkx2.5* expression domain appears enlarged although not to the same extent as that observed in zebrafish. However, the ratio of *Nkx2.5*-expressing cells to *HAND1*-expressing cells is markedly increased (Fig. 6) because of the observed decline of the *HAND1* expression domain. The enlarged *Nkx2.5* expression domain could be indicative of the general increase in anterior structures observed when RA signaling is blocked prior to gastrulation in *Xenopus* (Koide et al., 2001). Alternatively, the changes in *Nkx2.5* and *HAND1* expression may indicate a more general role for RA in patterning the lateral plate mesoderm.

The inability of the early antagonist treatment to generate a larger differentiated heart is clearly different than what is observed in zebrafish where the *cardiac myosin light chain 2* expression closely mirrors the *Nkx2.5* expression. At the time of cardiac differentiation, both the *Nkx2.5* expression domain and the expression of *cTnI* are much smaller than normal in *Xenopus*. Perhaps the additional cells expressing *Nkx2.5* lack another factor required for maintenance of *Nkx2.5* expression or the additional *Nkx2.5* expression is insufficient to cause the cells to change their fates. Our observation that *GATA-4* and *Nkx2.5* behave differently to alterations in RA signaling (Fig. 5) and that differentiation is only apparent where both are co-expressed would support this concept. Addition of exogenous RA results in expression of *cTnI* only in the anterior half of the heart-forming region, where *Nkx2.5* and *GATA-4* are co-expressed (compare Figs. 1 and 4). When embryos are treated with the AGN 194301, the observed dorsal spur of *Nkx2.5* expression reaches close to the developing somites (Fig. 4) but does not result in an expansion of *cTnI* expression. In these embryos, the *GATA-4* expression domain is restricted, although not eliminated (Fig. 4), as has been observed in *RALDH2* −/− mice (Niederreither et al., 2001) and in the quail dietary deficient model (Kostetskii et al., 1999). In these embryos, as well as control embryos, the overlap between the *Nkx 2.5* and *GATA-4* expression domains best predicts where *cTnI* will be expressed.

Retinoic acid signaling is required for rolling up of the heart tube

The most obvious defect observed in embryos where RA signaling was attenuated is the permanent lack of heart tube closure. Rolling up of the heart tube requires RA signaling between embryonic stage 14 and 18, well before the actual morphogenetic event (Fig. 4). A failure to transition from a sheet of cells to a heart tube has not been observed in other models of RA deficiency. This could imply an RA-sensitive event that is unique to *Xenopus*, although when comparable antagonist treatments have been done in chick, a widening of the myocardium can be seen, perhaps indicating a similar phenotype although tube closure was not directly assessed (Hochgreb et al., 2003). The mechanisms for transiting from a single fused heart primordium to a tube do appear to vary between model organisms. Zebrafish forms a cone structure (Glickman and Yelon, 2002), and mammals form both endocardial and myocardial tubes before fusion. These differences in cardiac morphogenesis may indicate unique molecular or morphogenetic events.

Alternatively, although the lack of tube formation in *Xenopus* appears novel, it may share common features with other RA deficiency experiments by representing a disruption in the normal timing of cardiac events. In both *RALDH2* and *RAR* knockout mice, premature differentiation of the myocardium is observed (Kastner et al., 1997; Niederreither et al., 1999). A plausible explanation of the failure to form a tube in *Xenopus* is that it represents a failure to progress through normal stages of morphogenesis, although precocious cardiac

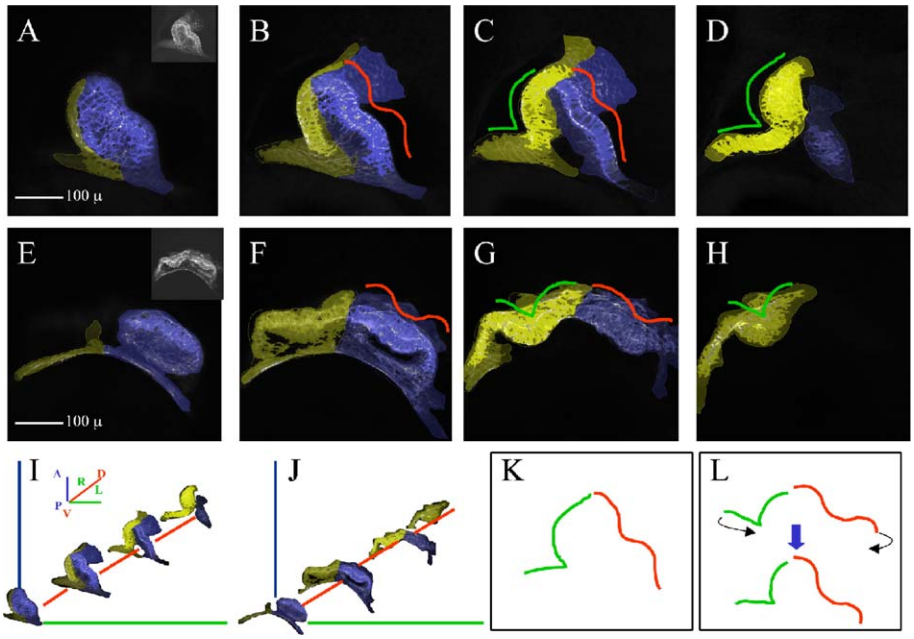

Fig. 9. Elements of the normal looping process are evident in the non-tubular hearts of RA-antagonist-treated embryos. Normal stage 33 embryos and RA-antagonist-treated embryos were fixed, immunolabeled with cardiac troponin I, imaged by confocal microscopy, and analyzed. Consistent characteristics among the groups were noted and are represented in the images of the individuals shown. Images A–D are a series of optical "thick sections"(ventral view) taken through a stage 33 normal *Xenopus* embryo (the inset in A is a compilation of the entire series). The series is presented in a ventral to dorsal progression as depicted in schematic I. At stage 33, the *Xenopus* heart field is just beginning tube closure (D), yet elements of looping have begun. (1) There is a ventral bulging of the heart field issue (evident by ventral–dorsal series A–D or I). (2) The asymmetric nature of the heart becomes more evident as it bends to form a "c-shaped" structure, curved to the embryos right (B, C). (3) The forming tube also rotates, positioning the original left heart field (pseudo-colored blue in series A–D, J) more ventrally and the original right heart field (pseudo colored yellow) more dorsally. These changes are illustrated in panels A–D with A, the most ventral sections, showing mainly left field (blue) progressing to D, the most dorsal sections, showing tissue only from the right field (yellow). The undeveloped future sino-atrial region remains evenly distributed across the right–left axis of the embryo, however, the right side is becoming more anterior than the left. In all species, the looping heart forms a series of complementary curves. For example, the outer portion of the "c shaped" curvature on the right side of the heart can be matched to the inner part of the "c-shaped" curve on the left side of the heart. The complementary curves are highlighted green and red on many of the panels and shown separately in panels K and L. Panels E–H (schematic J) are a ventral–dorsal series of the heart region of a 1 μM 194301-treated embryo fixed when untreated littermates reached stage 39. Inset E shows the entire z-series through the heart field compiled. Confocal imaging the myocardium of these embryos revealed that many characteristics of looping are present. (1) The heart fields of the RA-antagonist-treated embryos did not remain flat sheets. Although the most posterior (the would-be sino-atrial region of an earlier normal embryo) remained evenly distributed among the ventral–dorsal, the right–left, and the anterior–posterior axes (E, F), (2) anterior to this region, the left field (blue) assumed a ventral position (note presence in E, lack thereof in H) while the right field (yellow) assumed a more dorsal prominence (present in H and not E). (3) Additionally, the left and right fields make the appropriate "c-shaped" bends with respect to the embryo's midline (G, H). The most prominent left side curvature of F and the most prominent right side curvature of H are copy/pasted into cartoon L. By digitally swinging the bottom (anatomical posterior end) of the curved lines toward the anatomical midline, the complementary pair resembles the pattern of a stage 33 heart (L and K).

differentiation was not found (Table 2). Heart tube morphologies in antagonist-treated embryos range from failing to progress beyond a split ventral–lateral sheet of cells when exposed to high levels of antagonist to a heart tube that fails to execute the looping program when exposed to low levels of antagonist (Fig. 3). This range of defects can be viewed as snapshots of different steps along the morphogenetic pathway in normal *Xenopus* hearts (Kolker et al., 2000; Mohun et al., 2000). Supporting this concept is that the higher the dose of antagonist, the hearts appear to be stopped at earlier stages of morphogenesis (Fig. 7). In antagonist-treated embryos, individual cardiomyocytes also have an immature morphology (Fig. 7). A role for RA in controlling timing has been observed in the establishment of the segmentation clock in somites (Moreno and Kintner, 2004), the temporal control of transcription in the nervous system (Diez del Corral et al., 2003; Novitch et al., 2003) and in the limb bud (Mercader et al., 2000).

Patterning changes in the heart due to changes in RA signaling

In both chick and mouse, a model of heart tube patterning has been proposed that gives RA a role in specifying sino-atrial structures and that the source of RA is determined by the dynamic expression of *RALDH2* in the posterior and lateral plate mesoderm (Hochgreb et al., 2003; Xavier-Neto et al., 1999, 2000, 2001). When *Xenopus* embryos are treated with RA or RA antagonist, the position of the differentiated myocardium is altered along the anterior–posterior of the embryo. If differentiated myocardial tissue is seen in RA-treated embryos, the cTnI expressing region is at the anterior end of presumptive heart region (Fig. 1). However, we cannot conclude that anterior–posterior information has been altered within the developing myocardium. Examination of *Tbx5* and *Irx4* expression (Garriock et al., 2001; Horb and Thomsen, 1999), which could address this issue, was inconclusive as expression of both markers was very low in both

RA-treated and AGN-194301-treated embryos (data not shown).

RA signaling has been implicated in the formation of the left–right axis in several model systems, and abnormal heart looping is a consequence (Chazaud et al., 1999; Iulianella and Lohnes, 2002; Kawakami et al., 2005; Niederreither et al., 2001; Tanaka et al., 2005; Tsukui et al., 1999; Vermot et al., 2005; Wasiak and Lohnes, 1999). When we examined the cardiac morphology of AGN-194301-treated embryos at stage 32/34, the hearts appeared symmetrical (Fig. 2). However, when later stage embryos were examined using confocal microscopy, inflexions were observed in hearts that had even failed to fuse at the midline, indicating that at least some aspects of left–right asymmetry were still established even in these grossly abnormal hearts (Fig. 9).

In chick, flectin is asymmetrically expressed and is decreased and disorganized in quail embryos that lack RA due to dietary insufficiency (Linask et al., 2002; Tsuda et al., 1996). Although we see similar asymmetry in flectin expression during normal *Xenopus* heart development (Kolker, Dagle, and Weeks, in preparation), unlike the dietary restriction studies carried out in quail, we observe significant flectin expression in the AGN-194301-treated embryos (Fig. 8). We also observe that, even in the absence of full heart tube formation, some of the distinctive left–right axis changes are maintained (Fig. 9). This suggests that the early identification of sidedness is not lost when RA signaling is inhibited after gastrulation, but the completion of looping program is encumbered. The loss of laterality in dietary deficiency models is correlated with a loss of *Pitx2* and *nodal* expression, but not the expression of earlier genes that establish the left–right axis such as *activin receptor IIa*, *sonic hedgehog*, *caronte*, *Lefty-1*, and *fgf-8* (Romeih et al., 2003; Zile et al., 2000). Mice that express a dominant negative RARγ2 allele exhibit predominantly normal situs but have aberrant looping morphogenesis (Iulianella and Lohnes, 2002). In *RALDH2* −/− mice that have severely reduced levels of RA, there is also normal situs, but the heart is also impaired in its ability to loop (Niederreither et al., 2001). Together, these results point to a conserved role for retinoid signaling in the progress of cardiac morphogenesis or maintenance of a left–right pattern rather than a direct role in establishing left–right asymmetry. Some aspects of this model contradict results obtained using RA antagonists in mouse at the head fold stage where left–right asymmetry is blocked (Chazaud et al., 1999; Tsukui et al., 1999).

Consequences of blocking RA signaling for later cardiac development and physiology

RA-treated embryos develop an intact circulatory system as circulating blood could be observed, indicating that a heart tube does form and develop connections to the vascular system. Although a nub of beating tissue could be observed in the antagonist-treated embryos (Fig. 3B), there was a lack of circulating blood as would be predicted their cardiac morphology (Fig. 8). The reduction in size, position of the nub, and the lack of circulating blood were very similar to hearts seen in *Xenopus* embryos where Tbx5 activity is disrupted (Horb and Thomsen, 1999). This suggests that RA signaling and Tbx5 activity may be affecting common downstream events.

Interestingly, the nub had a heart rate that was reduced to about 50% of normal (Fig. 4). This is essentially the same as heart rate reductions seen in dietary deficiency models (Heine et al., 1985). It is unclear as to whether this difference in beating frequency is due to alterations in cardiac morphology or due to a direct effect on elements of the conduction system that are known to be sensitive to RA signaling (van Veen et al., 2002).

Taken together, these results demonstrate that RA signaling during a relatively narrow window of time is important for multiple aspects of cardiac development in *Xenopus*. Although the lack of heart tube formation is not observed in other model organisms, a common role for RA in controlling timing and differentiation in cardiogenesis may underlie the observed phenotype.

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References

Alsan, B.H., Schultheiss, T.M., 2002. Regulation of avian cardiogenesis by Fgf8 signaling. Development 129, 1935–1943.

Angelo, S., Lohr, J., Lee, K.H., Ticho, B.S., Breitbart, R.E., Hill, S., Yost, H.J., Srivastava, D., 2000. Conservation of sequence and expression of *Xenopus* and zebrafish dHAND during cardiac, branchial arch and lateral mesoderm development. Mech. Dev. 95, 231–237.

Breckenridge, R.A., Mohun, T.J., Amaya, E., 2001. A role for BMP signalling in heart looping morphogenesis in *Xenopus*. Dev. Biol. 232, 191–203.

Chazaud, C., Chambon, P., Dolle, P., 1999. Retinoic acid is required in the mouse embryo for left–right asymmetry determination and heart morphogenesis. Development 126, 2589–2596.

Chen, Y., Pollet, N., Niehrs, C., Pieler, T., 2001. Increased XRALDH2 activity has a posteriorizing effect on the central nervous system of *Xenopus* embryos. Mech. Dev. 101, 91–103.

Chen, T.H., Chang, T.C., Kang, J.O., Choudhary, B., Makita, T., Tran, C. M., Burch, J.B., Eid, H., Sucov, H.M., 2002. Epicardial induction of fetal cardiomyocyte proliferation via a retinoic acid-inducible trophic factor. Dev. Biol. 250, 198–207.

Diez del Corral, R., Olivera-Martinez, I., Goriely, A., Gale, E., Maden, M., Storey, K., 2003. Opposing FGF and retinoid pathways control ventral neural pattern, neuronal differentiation, and segmentation during body axis extension. Neuron 40, 65–79.

Drysdale, T.A., Tonissen, K.F., Patterson, K.D., Crawford, M.J., Krieg, P.A., 1994. Cardiac troponin I is a heart-specific marker in the *Xenopus* embryo: expression during abnormal heart morphogenesis. Dev. Biol. 165, 432–441.

Drysdale, T.A., Patterson, K.D., Saha, M., Krieg, P.A., 1997. Retinoic acid can block differentiation of the myocardium after heart specification. Dev. Biol. 188, 205–215.

Foley, A.C., Mercola, M., 2005. Heart induction by Wnt antagonists depends on the homeodomain transcription factor Hex. Genes Dev. 19, 387–396.

Garriock, R.J., Vokes, S.A., Small, E.M., Larson, R., Krieg, P.A., 2001. Developmental expression of the *Xenopus* Iroquois-family homeobox genes, Irx4 and Irx5. Dev. Genes Evol. 211, 257–260.

Ghatpande, S., Ghatpande, A., Zile, M., Evans, T., 2000. Anterior endoderm is sufficient to rescue foregut apoptosis and heart tube morphogenesis in an embryo lacking retinoic acid. Dev. Biol. 219, 59–70.

Glass, C.K., Rosenfeld, M.G., 2000. The coregulator exchange in transcriptional functions of nuclear receptors. Genes Dev. 14, 121–141.

Glickman, N.S., Yelon, D., 2002. Cardiac development in zebrafish: coordination of form and function. Semin. Cell Dev. Biol. 13, 507–513.

Harland, R.M., 1991. In situ hybridization: an improved whole-mount method for *Xenopus* embryos. Methods Cell. Biol. 36, 685–695.

Haselbeck, R.J., Hoffmann, I., Duester, G., 1999. Distinct functions for Aldh1 and Raldh2 in the control of ligand production for embryonic retinoid signaling pathways. Dev. Genet. 25, 353–364.

Heine, U.I., Roberts, A.B., Munoz, E.F., Roche, N.S., Sporn, M.B., 1985. Effects of retinoid deficiency on the development of the heart and vascular system of the quail embryo. Virchows Arch. B Cell Pathol. Incl. Mol. Pathol. 50, 135–152.

Hochgreb, T., Linhares, V.L., Menezes, D.C., Sampaio, A.C., Yan, C.Y., Cardoso, W.V., Rosenthal, N., Xavier-Neto, J., 2003. A caudorostral wave of RALDH2 conveys anteroposterior information to the cardiac field. Development 130, 5363–5374.

Hollemann, T., Chen, Y., Grunz, H., Pieler, T., 1998. Regionalized metabolic activity establishes boundaries of retinoic acid signalling. EMBO J. 17, 7361–7372.

Horb, M.E., Thomsen, G.H., 1999. Tbx5 is essential for heart development. Development 126, 1739–1751.

Iulianella, A., Lohnes, D., 2002. Chimeric analysis of retinoic acid receptor function during cardiac looping. Dev. Biol. 247, 62–75.

Jensen, A.M., Wallace, V.A., 1997. Expression of Sonic hedgehog and its putative role as a precursor cell mitogen in the developing mouse retina. Development 124, 363–371.

Jiang, Y., Drysdale, T.A., Evans, T., 1999. A role for GATA-4/5/6 in the regulation of Nkx2.5 expression with implications for patterning of the precardiac field. Dev. Biol. 216, 57–71.

Johnson, A.T., Klein, E.S., Gillett, S.J., Wang, L., Song, T.K., Pino, M.E., Chandraratna, R.A., 1995. Synthesis and characterization of a highly potent and effective antagonist of retinoic acid receptors. J. Med. Chem. 38, 4764–4767.

Kastner, P., Messaddeq, N., Mark, M., Wendling, O., Grondona, J.M., Ward, S., Ghyselinck, N., Chambon, P., 1997. Vitamin A deficiency and mutations of RXRalpha, RXRbeta and RARalpha lead to early differentiation of embryonic ventricular cardiomyocytes. Development 124, 4749–4758.

Kawakami, Y., Raya, A., Raya, R.M., Rodriguez-Esteban, C., Belmonte, J.C., 2005. Retinoic acid signalling links left–right asymmetric patterning and bilaterally symmetric somitogenesis in the zebrafish embryo. Nature 435, 165–171.

Keegan, B.R., Feldman, J.L., Begemann, G., Ingham, P.W., Yelon, D., 2005. Retinoic acid signaling restricts the cardiac progenitor pool. Science 307, 247–249.

Kelley, C., Blumberg, H., Zon, L.I., Evans, T., 1993. GATA-4 is a novel transcription factor expressed in endocardium of the developing heart. Development 118, 817–827.

Koide, T., Downes, M., Chandraratna, R.A., Blumberg, B., Umesono, K., 2001. Active repression of RAR signaling is required for head formation. Genes Dev. 15, 2111–2121.

Kolker, S.J., Tajchman, U., Weeks, D.L., 2000. Confocal imaging of early heart development in *Xenopus laevis*. Dev. Biol. 218, 64–73.

Kostetskii, I., Jiang, Y., Kostetskaia, E., Yuan, S., Evans, T., Zile, M., 1999. Retinoid signaling required for normal heart development regulates GATA-4 in a pathway distinct from cardiomyocyte differentiation. Dev. Biol. 206, 206–218.

Liberatore, C.M., Searcy-Schrick, R.D., Yutzey, K.E., 2000. Ventricular expression of tbx5 inhibits normal heart chamber development. Dev. Biol. 223, 169–180.

Linask, K.K., Yu, X., Chen, Y., Han, M.D., 2002. Directionality of heart looping: effects of Pitx2c misexpression on flectin asymmetry and midline structures. Dev. Biol. 246, 407–417.

Logan, M., Mohun, T., 1993. Induction of cardiac muscle differentiation in isolated animal pole explants of *Xenopus laevis* embryos. Development 118, 865–875.

Mercader, N., Leonardo, E., Piedra, M.E., Martinez, A.C., Ros, M.A., Torres, M., 2000. Opposing RA and FGF signals control proximodistal vertebrate limb development through regulation of Meis genes. Development 127, 3961–3970.

Mohun, T.J., Leong, L.M., Weninger, W.J., Sparrow, D.B., 2000. The morphology of heart development in *Xenopus laevis*. Dev. Biol. 218, 74–88.

Mohun, T., Orford, R., Shang, C., 2003. The origins of cardiac tissue in the amphibian, *Xenopus laevis*. Trends Cardiovasc. Med. 13, 244–248.

Moreno, T.A., Kintner, C., 2004. Regulation of segmental patterning by retinoic acid signaling during *Xenopus* somitogenesis. Dev. Cell 6, 205–218.

Munoz-Chapuli, R., Macias, D., Gonzalez-Iriarte, M., Carmona, R., Atencia, G., Perez-Pomares, J.M., 2002. The epicardium and epicardial-derived cells: multiple functions in cardiac development. Rev. Esp. Cardiol. 55, 1070–1082.

Nascone, N., Mercola, M., 1995. An inductive role for the endoderm in *Xenopus* cardiogenesis. Development 121, 515–523.

Niederreither, K., Subbarayan, V., Dolle, P., Chambon, P., 1999. Embryonic retinoic acid synthesis is essential for early mouse post-implantation development. Nat. Genet. 21, 444–448.

Niederreither, K., Vermot, J., Messaddeq, N., Schuhbaur, B., Chambon, P., Dolle, P., 2001. Embryonic retinoic acid synthesis is essential for heart morphogenesis in the mouse. Development 128, 1019–1031.

Nieuwkoop, P.D., Faber, J., 1994. Normal Table of *Xenopus laevis* (Daudin): A Systematical and Chronological Survey of the Development from the Fertilized Egg Till the End of Metamorphosis. Garland Pub, New York.

Novitch, B.G., Wichterle, H., Jessell, T.M., Sockanathan, S., 2003. A requirement for retinoic acid-mediated transcriptional activation in ventral neural patterning and motor neuron specification. Neuron 40, 81–95.

Pandur, P., Lasche, M., Eisenberg, L.M., Kuhl, M., 2002. Wnt-11 activation of a non-canonical Wnt signalling pathway is required for cardiogenesis. Nature 418, 636–641.

Raffin, M., Leong, L.M., Rones, M.S., Sparrow, D., Mohun, T., Mercola, M., 2000. Subdivision of the cardiac Nkx2.5 expression domain into myogenic and nonmyogenic compartments. Dev. Biol. 218, 326–340.

Romeih, M., Cui, J., Michaille, J.J., Jiang, W., Zile, M.H., 2003. Function of RARgamma and RARalpha2 at the initiation of retinoid signaling is essential for avian embryo survival and for distinct events in cardiac morphogenesis. Dev. Dyn. 228, 697–708.

Rones, M.S., McLaughlin, K.A., Raffin, M., Mercola, M., 2000. Serrate and Notch specify cell fates in the heart field by suppressing cardiomyogenesis. Development 127, 3865–3876.

Rosenthal, N., Xavier-Neto, J., 2000. From the bottom of the heart: anteroposterior decisions in cardiac muscle differentiation. Curr. Opin. Cell Biol. 12, 742–746.

Ross, S.A., McCaffery, P.J., Drager, U.C., De Luca, L.M., 2000. Retinoids in embryonal development. Physiol. Rev. 80, 1021–1054.

Sater, A.K., Jacobson, A.G., 1989. The specification of heart mesoderm occurs during gastrulation in *Xenopus laevis*. Development 105, 821–830.

Schneider, V.A., Mercola, M., 1999. Spatially distinct head and heart inducers within the *Xenopus* organizer region. Curr. Biol. 9, 800–809.

Schneider, V.A., Mercola, M., 2001. Wnt antagonism initiates cardiogenesis in *Xenopus laevis*. Genes Dev. 15, 304–315.

Stuckmann, I., Evans, S., Lassar, A.B., 2003. Erythropoietin and retinoic acid, secreted from the epicardium, are required for cardiac myocyte proliferation. Dev. Biol. 255, 334–349.

Sucov, H.M., Dyson, E., Gumeringer, C.L., Price, J., Chien, K.R., Evans, R.M., 1994. RXR alpha mutant mice establish a genetic basis for vitamin A signaling in heart morphogenesis. Genes Dev. 8, 1007–1018.

Tanaka, Y., Okada, Y., Hirokawa, N., 2005. FGF-induced vesicular release of

Sonic hedgehog and retinoic acid in leftward nodal flow is critical for left–right determination. Nature 435, 172–177.

Teng, M., Duong, T.T., Klein, E.S., Pino, M.E., Chandraratna, R.A., 1996. Identification of a retinoic acid receptor alpha subtype specific agonist. J. Med. Chem. 39, 3035–3038.

Teng, M., Duong, T.T., Johnson, A.T., Klein, E.S., Wang, L., Khalifa, B., Chandraratna, R.A., 1997. Identification of highly potent retinoic acid receptor alpha-selective antagonists. J. Med. Chem. 40, 2445–2451.

Tonissen, K.F., Drysdale, T.A., Lints, T.J., Harvey, R.P., Krieg, P.A., 1994. XNkx-2.5, a *Xenopus* gene related to Nkx-2.5 and tinman: evidence for a conserved role in cardiac development. Dev. Biol. 162, 325–328.

Tsuda, T., Philp, N., Zile, M.H., Linask, K.K., 1996. Left–right asymmetric localization of flectin in the extracellular matrix during heart looping. Dev. Biol. 173, 39–50.

Tsukui, T., Capdevila, J., Tamura, K., Ruiz-Lozano, P., Rodriguez-Esteban, C., Yonei-Tamura, S., Magallon, J., Chandraratna, R.A., Chien, K., Blumberg, B., Evans, R.M., Belmonte, J.C., 1999. Multiple left–right asymmetry defects in Shh(−/−) mutant mice unveil a convergence of the shh and retinoic acid pathways in the control of Lefty-1. Proc. Natl. Acad. Sci. U. S. A. 96, 11376–11381.

van Veen, T.A., van Rijen, H.V., Wiegerinck, R.F., Opthof, T., Colbert, M.C., Clement, S., de Bakker, J.M., Jongsma, H.J., 2002. Remodeling of gap junctions in mouse hearts hypertrophied by forced retinoic acid signaling. J. Mol. Cell. Cardiol. 34, 1411–1423.

Vermot, J., Llamas, J.G., Fraulob, V., Niederreither, K., Chambon, P., Dolle, P., 2005. Retinoic acid controls the bilateral symmetry of somite formation in the mouse embryo. Science 308, 563–566.

Wasiak, S., Lohnes, D., 1999. Retinoic acid affects left–right patterning. Dev. Biol. 215, 332–342.

Xavier-Neto, J., Neville, C.M., Shapiro, M.D., Houghton, L., Wang, G.F., Nikovits Jr., W., Stockdale, F.E., Rosenthal, N., 1999. A retinoic acid-inducible transgenic marker of sino-atrial development in the mouse heart. Development 126, 2677–2687.

Xavier-Neto, J., Shapiro, M.D., Houghton, L., Rosenthal, N., 2000. Sequential programs of retinoic acid synthesis in the myocardial and epicardial layers of the developing avian heart. Dev. Biol. 219, 129–141.

Xavier-Neto, J., Rosenthal, N., Silva, F.A., Matos, T.G., Hochgreb, T., Linhares, V.L., 2001. Retinoid signaling and cardiac anteroposterior segmentation. Genesis 31, 97–104.

Yelon, D., Ticho, B., Halpern, M.E., Ruvinsky, I., Ho, R.K., Silver, L.M., Stainier, D.Y., 2000. The bHLH transcription factor hand2 plays parallel roles in zebrafish heart and pectoral fin development. Development 127, 2573–2582.

Yutzey, K., Gannon, M., Bader, D., 1995. Diversification of cardiomyogenic cell lineages in vitro. Dev. Biol. 170, 531–541.

Zile, M.H., 2001. Function of vitamin A in vertebrate embryonic development. J. Nutr. 131, 705–708.

Zile, M.H., Kostetskii, I., Yuan, S., Kostetskaia, E., St. Amand, T.R., Chen, Y., Jiang, W., 2000. Retinoid signaling is required to complete the vertebrate cardiac left/right asymmetry pathway. Dev. Biol. 223, 323–338.